Detection of recombinations between c-myc and immunoglobulin switch α in murine plasma cell tumors and preneoplastic lesions by polymerase chain reaction

(protooncogene/chromosomal translocation/gene rearrangement/plasmacytomagenesis/BALB/c mouse)

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ABSTRACT Virtually all murine plasmacytomas carry chromosomal translocations that activate c-myc. The predominating (≈90%) c-myc-activating chromosomal translocation in pristane (2,6,10,14-tetramethylpentadecane)-induced plasmacytomas in BALB/c mice is a reciprocal translocation t(12;15) in which an immunoglobulin heavy-chain switch sequence is joined to the 5' region of c-myc. The most common switch region involved is Sα. We developed a direct PCR method to screen for recombinations between c-myc and Sα. The critical step in establishing the method was the cloning and sequencing of the 5' flank of Cα, a region with a reduced number of switch repeats that is much more favorable for designing specific PCR primers than the highly repetitive Sα region. In applying this PCR method, we detected translocation-specific junction fragments in transplanted (10/16, 63%) and primary (5/15, 33%) plasmacytomas. Moreover, the sensitivity of a nested version of this technique allowed us to discern rare (t(12;15))s in BALB/c mice in the preneoplastic stage of plasmacytomagenesis (8/20 mice, 40%) as early as 30 days after administration of pristane. We conclude that t(12;15) is the probable primary, if not initiating, oncogenic step in plasmacytomagenesis.

Plasmacytomas (PCTs) induced in BALB/cAnPt mice by the intraperitoneal (i.p.) injection of pristane (2,6,10,14-tetramethylpentadecane) (1) carry reciprocal chromosomal translocations t(12;15), t(6;15), or t(15;16) that are associated with the activation of the c-myc protooncogene (2). In the t(12;15) which occurs in 90% of the tumors the near 5' flanking region, the first exon or part of the first intron of c-myc is disrupted and joined head to head with genes in the immunoglobulin heavy chain (IgH) gene complex (3–6). The most common immunoglobulin gene breakpoint is in either the α-chain switch (Sα) region (7) or the flanking region just 5' of exon 1 of the α-chain constant region (5'-Cα) (8). That region (Sα/5'-Cα) is involved in 80% (28/35) of all of the PCTs with t(12;15)s that have been molecularly characterized (reviewed in 9). The goal of the present study was to take advantage of these common breakpoints in Sα/5'-Cα and devise a PCR method for detecting illegitimates—i.e., nonhomologous-recombinations between Sα/5'-Cα and c-myc. We describe here a direct PCR technique that detects 63% (10/16) of all translocation breakpoints in transplanted PCTs that are known to involve the Sα/5'-Cα region. We have applied this methodology to detect similar translocations in primary tumors and in the very early preneoplastic stages of PCT development.

MATERIALS AND METHODS

Induction, Diagnosis, and Transplantation of PCTs. Primary PCTs were induced by i.p. administration of plasmacytoma (2,6,10,14-tetramethylpentadecane; Aldrich) in conventionally maintained BALB/cAnPt inbred or susceptible BALB/cAnPt.DBA/2N-Idh1-Pep3 congenic mice (M.P., unpublished data). PCTs were diagnosed and transplanted as described (9). The mice were bred and maintained at Hazleton Laboratories, Rockville, MD under National Cancer Institute contract (NOI-CB-21075).

Preparation of DNA Templates for PCR. High molecular weight DNA was prepared from tissues that had been snap-frozen in liquid nitrogen or from cultured cells according to a standard phenol/chloroform extraction protocols that include digestion with proteinase K (100 μg/ml, Boehringer, Mannheim) and RNase A (40 μg/ml, Sigma). For rapid preparation of DNA from single cell suspensions and tissues, a lysis buffer consisting of 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl2, 0.5% Tween 20, and proteinase K at 50–300 μg/ml was employed. Cells or tissues were incubated in lysis buffer at 60°C for 4–16 h and then incubated at 90°C for 30–60 min to inactivate proteinase K. This simple in-tube protocol usually results in the release of DNA with an A260/A280 ratio=1.4 that serves effectively as a PCR template.

PCR Primers. PCR primers were initially designed by assessing available sequence data. Later, the primer analysis software Oligo 4.1 (National Biosciences, Plymouth, MN) was employed. Altogether, 15 primers for exon 1 and intron 1 of c-myc and 23 for the Sα/5'-Cα region were tested. Table 1 gives the sequences for those primers found to be most reliable throughout the study. Position numbers in the Sα/5'-Cα region refer to the aligned sequence of Sα (GenBank entries MUSREQU and MUSIGCD41) and 5'-Cα (this paper) with the first base in MUSREQU being position 1. Oligonucleotides were prepared on a DNA synthesizer (Applied Biosystems) or purchased from Bioserve (Laurel, MD).

PCR Amplification. PCR reactions were run with reagents from Boehringer Mannheim according to their recommendations. In most cases a hot-start technique using wax pearls (Perkin–Elmer) was employed. Amounts of 10 ng to 1 μg of DNA were amplified in a final volume of 100 μl of PCR buffer consisting of 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, gelatin at 0.1 mg/ml, each primer at 0.5 μM, each dNTP at 200 μM (Boehringer Mannheim), and 2.5 units of Taq DNA polymerase. The PCR reaction was performed in

Abbreviations: MOPC, mineral oil-induced plasmacytoma; TEPC, tetramethylpentadecane-induced plasmacytoma; PCT(s), plasmacytomats; IgH, immunoglobulin heavy chain; IgHα, immunoglobulin heavy chain α; Sα, switch region of α chain gene; Cα, immunoglobulin α-chain constant region; 5'-Cα, 5' flanking region of Cα; t(12;15)(s), reciprocal translocation(s) involving band F1 on chromosome 12 and band D2 on chromosome 15; t(6;15), reciprocal translocation involving band C2 on chromosome 6 and band D2 on chromosome 15; t(15;16), reciprocal translocation involving band D2 on chromosome 15 and band B1 on chromosome 16.

*The sequence reported in this paper has been deposited in the GenBank data base (accession no. L13590).
a PTC-100 programmable thermal controller (MJ Research, Watertown, MA) for 40 cycles at constant primer annealing and DNA melting times but steadily increasing DNA extension times. The following conditions were chosen: (i) initial denaturation at 95°C for 5 min; (ii) incubation for 10 cycles at 65°C for 15 sec, 72°C for 15 sec, and 95°C for 15 sec; (iii) followed by 10 cycles each at 65°C for 30 sec, 72°C for 60 sec, and 95°C for 90 sec extension time; (iv) final extension at 72°C for 10 min; and (v) indefinite holding at 4°C. The annealing temperature (T_a) needed to be optimized for each primer pair and varied between 52°C and 68°C. However, most of the routine screening experiments were done at T_a = 65°C. The second round of PCR was performed as nested PCR on a 5-μl aliquot of the first round PCR mixture. In most cases a primer pair was chosen that would be complementary as far as possible at the 5’ and 3’ ends of the first PCR product. PCR fragments in the range of 0.5–3 kb could be amplified by employing this protocol. PCR products from one- and two-round PCRs were fractionated by standard electrophoresis on 1.5–4% agarose (electrophoresis grade, BRL) gels.

Cloning and Sequencing of PCR Products. PCR products were ligated into pCRII cloning vectors and cloned in Escherichia coli (Invitrogen) without prior purification of the PCR product in most cases. PCR fragments were purified with Magic PCR Preps (Promega) when the initial cloning experiment was unsuccessful. Plasmid DNA was prepared from bacterial overnight cultures by using Magic Maxipreps (Promega). Dideoxy-termination DNA sequencing was performed on double-stranded DNA by using the Sequenase kit (United States Biochemical) according to the instructions of the supplier.

Cloning and Sequencing of the 5’-C_a. A rearranged 5.4-kb IgA fragment from the Abelson virus-induced PCT ABPC 60, pAB2.1, was cloned from a bacteriophage genomic library. Restriction mapping and partial sequence analysis of pAB2.1 showed that one end of the insert contained germ-line IgA sequences including the 5’ flank of C_a, whereas the other end of the clone mapped within the chromosome 15 c-myc/Pvt-1 amplicon (J.S., unpublished data). Clone pAB2.1 was chosen to sequence the 5’-C_a region, using the Sequenase method as described above.

RESULTS

PCR Amplification of c-myc/IgH disadv. In preliminary studies we found that the highly repetitive tandem pentameric sequence of S_a (CTGGR) is a major obstacle for specific primer annealing and extension in this region. The S_a sequence as originally determined (10) has been recently extended (11). However, the nucleotide sequence bridging S_a and C_a—i.e., 5’-C_a—which we report here (Fig. 1), had not been previously determined. This region contains far fewer repetitive switch pentamers than S_a (see Fig. 3C) and therefore provides primer sites that are more suitable for initiating PCR-driven DNA synthesis through the translocation breakpoints into c-myc. To establish the PCR technique a panel of 16 transplanted PCTs with molecularly verified breakpoints associated with the S_a region were selected (8). When the primers shown in Table 1 were used, c-myc/IgH_junction fragments were amplified in 10 of 16 (62%) transplanted tumors (Table 2), and the products were characterized by DNA sequencing (Table 3). Amplified products were not detected in 6 of 16 PCTs; MOPC 41, MOPC 46B, TEPC 15, TEPC 609, SAM 368, and BAL 17. The reasons for that are presently unclear. The PCR-generated junction fragments of five PCTs were compared with those previously described by cloning and sequencing of recombinant joints. For the remaining five junction fragments generated by PCR previous sequence data were not available. PCR analysis confirmed the previously determined translocation breakpoints for MOPC 167 (12, 13), TEPC 1165 (14), and HOPC 1 (6). Based on the better definition of the S_a/5’-C_a region, the PCR breakpoints for MOPC 315 (13) and XRPC 24 (15) in c-myc were located 6 and 4 bp 5’ of the sites previously described, respectively.

We extended this analysis to 15 primary PCTs and found five tumors (33%) that yielded junction fragments; the sequences of these five are given in Table 3. The t(12;15) of one tumor (Co.1) was confirmed cytogenetically (F. Wiener, unpublished result). The result of studies on primary and transplant PCTs indicated that PCR analysis could be effective in determining when the t(12;15) take place in

Table 1. PCR primers

<table>
<thead>
<tr>
<th>Primer</th>
<th>Position</th>
<th>Sequence (5’→3’)</th>
<th>Length, nt</th>
</tr>
</thead>
<tbody>
<tr>
<td>myc1</td>
<td>532–564*</td>
<td>TCTTATCAGCGAATCGAGTCCCTTCCCTCCCT</td>
<td>31</td>
</tr>
<tr>
<td>myc2</td>
<td>1625–1659*</td>
<td>CCAATCTCAAGAATCTCCAGTACCTCCTTGTCCTCCTCAT</td>
<td>35</td>
</tr>
<tr>
<td>myc3</td>
<td>1677–1711*</td>
<td>AGGGATCCCGCAGGCCTCAAGAGTTAGG</td>
<td>39</td>
</tr>
<tr>
<td>a1</td>
<td>2109–2133*</td>
<td>AGCTACAGCTGACCTAGGCGAGCCCTC</td>
<td>25</td>
</tr>
<tr>
<td>a2</td>
<td>2363–2381*</td>
<td>TGTGAGCAAGCCAGGTTA</td>
<td>19</td>
</tr>
<tr>
<td>a3</td>
<td>3170–3189</td>
<td>TCACAAACCAAGACCAGAGAC</td>
<td>20</td>
</tr>
<tr>
<td>a4</td>
<td>3365–3383</td>
<td>GCTTCAGACCCACCACCTCC</td>
<td>19</td>
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<td>a5</td>
<td>3589–3608*</td>
<td>TTGTTGGGATGAGTACAGGC</td>
<td>20</td>
</tr>
<tr>
<td>a6</td>
<td>3746–3768</td>
<td>CCAACAGGTCCTGCTGTATATAC</td>
<td>23</td>
</tr>
<tr>
<td>a7</td>
<td>113–132</td>
<td>ATCAGGAGCAGGGATATC</td>
<td>20</td>
</tr>
</tbody>
</table>

*Base pair number in GenBank file MUSCMYC1.
†Base pair number in S_a/5’-C_a in aligned sequence (this paper).
‡Base pairs 113–132 in GenBank file MUSCMYC2 (complementary to a sequence 66 bp into C_a).

![Fig. 1. DNA sequence of 5’-C_a. The consensus S_a pentamers CTGAG and CTGGG are underlined. A noteworthy region that consists of the sixfold uninterrupted repeat of the 20-bp sequence CTGGG CTAGG CTTRR TTAGT (nt 114–234) and also contains typical S_a repeats is given in italics.](image-url)
plasmacytomagenesis. Therefore, nested PCR was applied to detect t(12;15)s in the very tissue where PCTs develop, the peritoneal oil granuloma. The intestinal mesenteric tissue of four mice 110 days after the injection of pristane was cut into five to seven sectors and then used for PCR amplification and histological examination. Three to seven plasmacytic foci per mouse were found. One mouse yielded a junction fragment (Table 2) indicating that the plasma cells in foci had t(12;15)s. These observations were then extended to the very early oil granuloma tissue at day 30 after pristane in 20 BALB/c mice. Surprisingly, 8 mice yielded c-myc/IgHα junction fragments (Table 2); in 6 of 8 mice the recombination joint was found in only one mesenteric sector and in 2 of 8 mice, in two sectors. Thus the majority of the sectors investigated remained negative. These results were confirmed by repeat experiments in which the pattern of positives and negatives was reproduced. The results of all PCR analyses are summarized in Table 2.

### Table 2. Summary of PCR results

<table>
<thead>
<tr>
<th>Tissue tested</th>
<th>No. of mice</th>
<th>PCR fragments amplified</th>
<th>Days after pristane</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transplanted PCTs*</td>
<td>16</td>
<td>10</td>
<td>62</td>
</tr>
<tr>
<td>Primary PCTs†</td>
<td>13</td>
<td>4</td>
<td>31</td>
</tr>
<tr>
<td>Primary PCTs‡</td>
<td>2</td>
<td>1</td>
<td>185</td>
</tr>
<tr>
<td>Oil granulomas§</td>
<td>4</td>
<td>1</td>
<td>110</td>
</tr>
<tr>
<td>Oil granulomas¶</td>
<td>20</td>
<td>8</td>
<td>40</td>
</tr>
</tbody>
</table>

* c-myc-IgHα-positive transplanted PCTs in BALB/cAnPt mice; NA, not applicable.
† Primary PCTs in C.D2-Idh-Pep3 mice.
‡ Primary PCTs in BALB/cAnPt mice.
§ Oil granulomas from tumor-free C.D2-Idh-Pep3 mice.
¶ Oil granulomas from tumor-free BALB/cAnPt mice.

### Table 3. Sequences adjoining chromosomal breakpoints on chromosome 12*

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Sα/5'-Cα</th>
<th>Tumor</th>
<th>c-myc</th>
<th>Breaksite position*</th>
<th>PCR primers†</th>
</tr>
</thead>
<tbody>
<tr>
<td>MOPC 167‡</td>
<td>atccaaActtcagttt</td>
<td>MOPC 173‡</td>
<td>ctcgctcgcctag</td>
<td>gggtCtaacccgggag</td>
<td>2252</td>
</tr>
<tr>
<td>MOPC 315‡</td>
<td>cccagctctctag</td>
<td>MOPC 511‡</td>
<td>cagctgctgctag</td>
<td>tttgaaggctgcc</td>
<td>930</td>
</tr>
<tr>
<td>TEPC 601†</td>
<td>agcctaaccA-tc</td>
<td>TEPC 1017†</td>
<td>tcttaCtaccttagg</td>
<td>ttcttggggcttg</td>
<td>1044</td>
</tr>
<tr>
<td>TEPC 1165†</td>
<td>gtcctagctgcctag</td>
<td>XRPC 24†</td>
<td>gtcctagcagacc</td>
<td>ttcggtgcctgggctct</td>
<td>2415</td>
</tr>
<tr>
<td>HOPC 11‡</td>
<td>acgctgctgctca</td>
<td>HOPC 6‡</td>
<td>acgctgctgctca</td>
<td>tccgctgctgctgctccag</td>
<td>3334</td>
</tr>
<tr>
<td>4122‡</td>
<td>cccagccagctcat</td>
<td>4124‡</td>
<td>tccatctgctgctag</td>
<td>tccgctgctgctgctccag</td>
<td>2268</td>
</tr>
<tr>
<td>4128‡</td>
<td>aagagcaaggggctcag</td>
<td>4132‡</td>
<td>cagctgctgctgctgctccag</td>
<td>ttcggtgcctgggctct</td>
<td>3369</td>
</tr>
<tr>
<td>Co.1†</td>
<td>gagaatataaaccag</td>
<td>Co.2†</td>
<td>gagaatataaaccag</td>
<td>ttcggtgcctgggctct</td>
<td>1711</td>
</tr>
</tbody>
</table>

† Transplanted PCT.
‡ Three breaksites are possible, and the most 5′ one with respect to c-myc is given.
§ Two breaksites are possible and the most 5′ one with respect to c-myc is given.
¶ Primary PCT.

DISCUSSION

Detection of c-myc/IgHα Recombinations by PCR. The PCR method described in this paper has the potential to detect all translocation breakpoints in c-myc that occur in the region of the gene indicated in Fig. 2A as the 5′ and 3′ limit of breaksites. In contrast, the method is limited to segments of the IgH locus in several ways: (i) Only breaksites in IgHα can be identified, although other regions of the IgH gene cluster, most notably Sμ, Sν2a, and Sν2b, are known to be utilized in some PCTs (Fig. 3A). (ii) Within the entire IgHα locus only breakpoints in Sα/5'-Cα can be detected. This restriction potentially misses other sites in IgHα such as in Cν and 5′ of Sα. (iii) The repetitive sequence of the Sα/5'-Cα region (Fig. 3C) limits the design of specific oligonucleotides to the 5′-Cα.
region, whereas all $S_a$ primers tested represent consensus sequences. However, using consensus primers in a highly repetitive $S_a$ region invites mispriming and PCR artefacts. We were able to design only a single primer (a1; Table 1) for the 3-kbp $S_a$ region (combined GenBank entries MUSS-REGU and MUSIGCD41) that worked reliably. It is noteworthy, though, that so far direct PCR in immunoglobulin S regions has been restricted to the use of primers that are complementary to the nonrepetitive flanking regions (18–20). Our data, however, show that primer complementary to sequences within an $S$ region can on some occasions be employed.

**Isotype Switching and t(12;15).** It is not known whether $t(12;15)$s are mediated by aberrant switch recombinase activities. However, the distribution of chromosomal breakpoints along $S_a$/5'-$C_a$, either within or downstream of physiologic switch recombination sites, suggests a mechanism of recombination that involves switch recombinase-mediated cuts within the $S_a$ acceptor site region followed by deletions that move the breaksites further toward $C_a$ (Fig. 3B). The hypothesis that switch recombinase is involved in $t(12;15)$ is supported by the following facts: First, switching is not confined to the functional chromosome but occurs on both chromosomes, often at the same IgH region (21, 22). Thus switching to IgH$_a$ on the productive allele may occur in parallel to switching to IgH$_a$ on the nonproductive allele. It is feasible that during this process the latter allele is rendered accessible to recombination with c-myc. Second, the ability for such illegitimate recombination to occur may somehow be related to the imprecision of the switching process, which is known to be particularly variable for the nonproductive IgH gene locus (23). Third, c-myc contains numerous $S_a$ (and $S_a$) pentamers in its 5' region (Fig. 2C) that had been noticed previously (6, 24). It is conceivable that these repetitive pentamers somehow facilitate recombination with $S$ regions as suspected early on (24). However, the excess of $S_a$ pentamers in the region of c-myc that is indicated in Fig. 2C is modest; i.e., the motifs GGGGT, CTGGG, and CTGAG occur 3–4 times more frequently than expected, and so far we have no proof for their involvement. Short direct repeats distinct from $S_a$ pentamers may also increase the likelihood for c-myc/IgH$_a$ rearrangements. In this respect, the excess of short recombinogenic motifs in the 5' region of c-myc such as the tetrmeric sequences GAGG (13, 25), CCCT (26), CGGC (27), and the pentameric GC stretches CGCGG/CGGCC (refs. 28 and 29; Fig. 2C) is suggestive. Furthermore, the occurrence of $\chi$-like sequences (CCWCCWGC) in c-myc which have been causally related to chromosomal translocations involving the protooncogene bcl-2 (30) is also noted, but its significance remains unclear.

**Time and Origin of t(12;15).** Preneoplastic proliferative foci of plasma cells have been identified morphologically in oil granulomatous tissues, but most of these occur beyond day 75 after injection of pristane. The surprising finding in this study was the presence of $t(12;15)$-specific PCR junction fragments in some sectors of the mesenteric oil granulomas of BALB/c mice 30 days after pristane. This provides compelling evidence that c-myc activating chromosomal translocations are a very early if not initiating step in plasma cell tumor formation. The restricted location of these cells to discrete sectors of the mesentery suggests that focal expansion of $t(12;15)$-positive cells, rather than diffuse infiltration, occurs at early stages of plasmacytogenesis. The remarkably large number of recombinations at 30 days (8/20 mice, 40%) suggests, however, that many of these translocated cells do not successfully evolve into PCTs and that chromosomal translocations are not the limiting step in BALB/c plasmacytogenesis. Subsequent events appear to be es-
sential for the development of a neoplastic state. The results presented here add to the list of PCR-detectable protooncogene-activating events that occur early in neoplastic development of other hematological malignancies—e.g., chromosomal translocations t(14;18) that activate bcl-2 in humans (31) and insertional mutagenesis in c-myc in mice (32).

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